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# The Effects of Charge Separation in Quaternary Ammonium, DABCO-Containing Polymers on In Vitro Toxicity and Gene Delivery

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## ABSTRACT

Polycation materials have recently emerged as promising systems for the delivery of genetic material. In this study, several DABCO (1,4-diazabicyclo[2.2.2]octane) polymers are investigated for their ability to bind and deliver plasmid DNA (pDNA) into mammalian cells. The DABCO polymers are synthesized by copolymerization of DABCO with 1,3-dibromopropane (D3), 1,4-dibromobutane (D4), 1,6-dibromohexane (D6), 1,8-dibromooctane (D8), and 1,10-dibromodecane (D10) to form a series of quaternary ammonium polymers with increasing charge separation. Gel retardation experiments reveal that each polymer (D3-D10) binds pDNA above a charge ratio of 1.0 (polymer + / pDNA -). The polycations are examined for *in vitro* transfection efficiency and toxicity in BHK-21 cells. Results of the transfection experiments indicate that the D6 polymer had the highest transfection efficiency. Although all of the polymers are shown to have some toxicity, the D8 and D10 polymers are more toxic to BHK-21 cells; approximately 30% of the cells survive at a charge ratio of 5 +/- as compared to the D3, D4, and D6 polymers where survival rates are about 80%.

## INTRODUCTION

Cationic polymers are currently being studied as alternatives to viral systems for the delivery of therapeutic genes. Advantages of using nonviral delivery vectors over their viral counterparts include low immunogenicity, noninfectivity, and virtually no limit to the size of the foreign gene that these vectors can carry [1-3]. Polycations have the ability to self-assemble with DNA and condense it into small particles that have been denoted as polyplexes. Polyplexes have been shown to deliver DNA into cultured cells through the endocytotic pathway. Several studies on polymeric delivery vectors have indicated that small changes in the structures of polymeric vectors play a significant yet undetermined role in the delivery efficiency and toxicity of these systems [4-7]. For example, previous experiments conducted by our group have indicated that the charge separation in β-cyclodextrin-containing polymers has a considerable effect on both the toxicity as well as the transfection efficiency of the delivery vectors in mammalian cell lines [6]. In the current study, several DABCO-containing polymers with increasing charge separations (Figure 1) have been created to further elucidate the effect of the charge separation within polycation delivery vectors. DABCO-based polymers have been formerly studied for other applications such as structure-directing agents in zeolite synthesis [8,9]. Here, their effectiveness as gene delivery agents is considered.

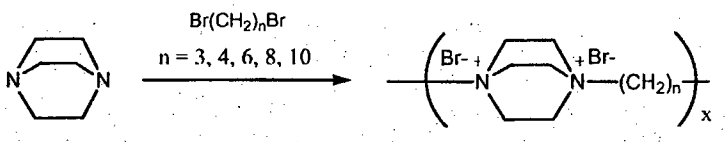


**Figure 1.** The structure of the DABCO polymers used in this study. The separation of the charge centers was varied by 3, 4, 6, 8, and 10 methylene units through copolymerization of DABCO with the corresponding dibromoalkane.

## EXPERIMENTAL DETAILS

### Polymer synthesis

All chemicals used in the synthesis of the polymers were obtained from Aldrich Chemical Company unless otherwise indicated. The polymers were synthesized by the stoichiometric copolymerization of DABCO and the corresponding dibromoalkane (1,3-dibromopropane, 1,4-dibromobutane, 1,6-dibromohexane, 1,8-dibromooctane, or 1,10-dibromodecane) in either dimethylformamide or dimethylsulfoxide and stirred at a temperature between 60°C and 70°C for 24 hours. After 24 hours, each product was purified by pipetting the reaction mixture into a Spectra/Por 1000 MWCO dialysis membrane and exhaustively dialyzing the product in Nanopure water for approximately 48 hours. The dialyzed product was then lyophilized to dryness.

### Polymer Characterization

Polymer molecular weights were determined by static light scattering. The polymers were analyzed on a Hitachi D6000 HPLC system equipped with a ERC-7512 RI detector, a Precision Detectors PD2020/DLS and a PL Aquagel-OH 30 column using 0.8M ammonium acetate pH = 2.8 with formic acid as the eluant at a 0.7 mL/min flow rate. The refractive index increment values were determined at 25°C (633 nm) in the same eluant above.

### Gel Retardation Experiments

Each polymer was examined for its ability to bind pDNA through gel electrophoresis experiments as previously described [10]. 1 μg of pGL3-CV (10 μL of a 0.1 μg/μL in DNase free water) was mixed with an equal volume of polymer at the appropriate charge ratios. Each solution was incubated for approximately 30 minutes. 2 μL of loading buffer was added to each sample and then 10 μL of each sample was pipetted into the wells of a 0.6% agarose gel containing 6 μg of ethidium bromide/100 mL TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and electrophoresed.

### Cell Culture Experiments

Plasmid DNA, pGL3-CV (Promega, Madison WI), containing the luciferase gene under the control of the SV40 promoter was amplified by *Escherichia Coli* strain DH5α and was then purified using Qiagen's Endotoxin-free Megaprep kit (Valencia, CA). 60 μL of cationic polymer

dissolved in DNase free water (Gibco BRL) was added to 60 µl of pDNA (0.1 mg/mL in DNase free water at charge ratios of 5, 10, 15, 20, 25, 30 (polymer + / pDNA – ). The mixtures were incubated for 30 minutes before cell transfection. BHK-21 cells were purchased from ATCC (Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 units/mg penicillin, 100 µg/mL streptomycin, and 0.25 µg/ml amphotericin at 37°C and 5% $CO_2$. Media and supplements were purchased from Gibco BRL (Gaithersburg, MD). BHK-21 cells were plated at 50,000 cells per well in 24 well plates and incubated for 24 hours. Cells were transfected with 1 µg of pGL3-CV complexed with each of the polymers above (D3, D4, D6, D8, D10) at the various charge ratios in triplicate in serum free media. After 4 hours, 800 mL of DMEM was added to each well. 24 hours after transfection, the media was replaced with 1 mL of DMEM. 48 hours after transfection, cell lysates were analyzed for luciferase protein activity with results reported in relative light units (RLUs). Toxicities were determined by the Lowry protein assay as previously described [6,10].

## DISCUSSION

### Polymer Synthesis and Characterization

The DABCO polymers were synthesized as previously described through quaternization of the tertiary amines on the DABCO units with the corresponding dibromoalkane [8,9]. As shown in Table 1, the molecular weight values and degrees of polymerizations are all within normal parameters for this class of polymers as determined by static light scattering [8]. The data given in Table 1 show degrees of polymerization for these polymers between 10 and 20 with the D4 polymer having the largest chain length and the D10 polymer having the lowest degree of polymerization.

Gel retardation experiments were completed for each DABCO polymer (D3-D10) and reveal that all polymers bound pDNA (a requirement for cellular uptake) as expected above a charge ratio of 1.0 +/-. An example of this experiment is shown in Figure 2, where the naked pDNA migrates in response to the electric field but when pDNA is bound by the polymer at and above a charge ratio of 1.0, the migration of pDNA is retarded indicating polymer binding. This experiment is a qualitative determination of pDNA binding but does not give quantitative information on polymer binding strength.

**Table 1.** The refractive index increment, molecular weight, polydispersity, and degree of polymerization data for the DABCO based polymers as determined by static light scattering.

| Polymer | D3 | D4 | D6 | D8 | D10 |
|---|---|---|---|---|---|
| dn/dc | 0.138 | 0.138 | 0.138 | 0.146 | 0.163 |
| Mw (kDa) | 5.10 | 6.70 | 4.82 | 6.44 | 4.41 |
| Mw/Mn | 1.22 | 1.34 | 1.22 | 1.29 | 1.23 |
| Degree of Polymerization | 16 | 20 | 14 | 17 | 11 |

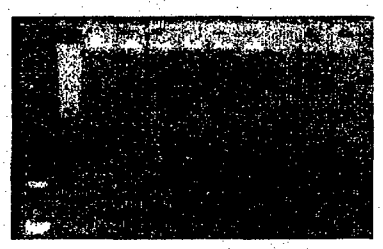


**Figure 2.** Example of gel retardation results. In this experiment, the D4 polymer was tested for binding to pDNA. The numbers, 0.5-5.0, represent the charge ratio (polymer + / pDNA – ) used in the analyses. Polymers D3-D10 were all examined similarly, and bound pDNA at and above a charge ratio of 1.0 +/- (electrophoresis results were very similar to what is shown here).

**Transfection and Toxicity Experiments**

The polyplexes formed by all of the DABCO polymers are able to transfect BHK-21 cells *in vitro* at several charge ratios. Transfection experiments reveal that the polymers have some toxicity and complete cell death is found above a charge ratio of 20 +/- as determined by the Lowry protein assays. As shown in Figure 3, the D3, D4, and D6 polymers are only slightly toxic at a charge ratio of 5 +/- with cell survivals of approximately 80%, and the D4 gives the lowest toxicity. Polymers D8 and D10 are found to have the highest toxicity at all charge ratios. At a charge ratio of 5 +/-, only 30% of the cells survived for the polymers D8 and D10. This result is inconsistent with what was observed in a previous structure-property study conducted in our lab. Using β-cyclodextrin polymers (βCDPs), the polyplexes formed by the polymer with a charge separation of 8 methylene units had the lowest toxicity [6].

All of the DABCO polyplexes are able to transfect BHK-21 cells with differing degrees of efficiency as determined by luciferase protein activity. As shown in Figure 4, the transfection is the most efficient for all of the polyplexes at a charge ratio of 10 +/-. The highest transfection efficiency is achieved by the D6 system. This result is consistent with the βCDPs in the previously mentioned study where a charge separation of 6 methylene units between the β-cyclodextrin monomers had the highest transfection efficiency in BHK-21 cells.

Although the current study was carried out in order to verify the relationship between charge separation, toxicity, and transfection efficiency, the DABCO based polymers are not ideal models. Even though the charge separation can be modified similar to the βCDPs, using different dibromoalkane spacers, each DABCO polymer contains 2 quaternary ammonium charges closely spaced on the DABCO units as pictured in Figure 1. However, in the βCDPs (Figure 5), the amidine charge centers are spaced far apart by both the β-cyclodextrin monomers as well as by the various lengths of methylene units. These structural differences as well as variations in the water solubility could account for the disagreement in toxicity that was observed between the βCDPs and the DABCO polymers.

**CONCLUSIONS**

Many questions remain unanswered regarding the structure-property relationships for polycationic gene delivery vectors. The current study was completed in order to reveal how the

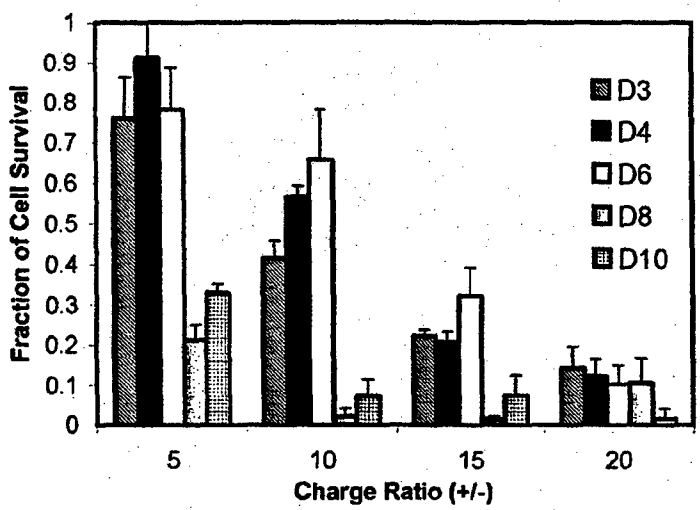


**Figure 3.** Comparison of the relative toxicities of the DABCO polyplexes at charge ratios of 5, 10, 15, and 20 +/- with BHK-21 cells. Cell Survival was determined by assaying for total protein concentration and normalizing each sample with the protein concentration for untransfected cells. The data are reported as a mean ± SD of three samples.

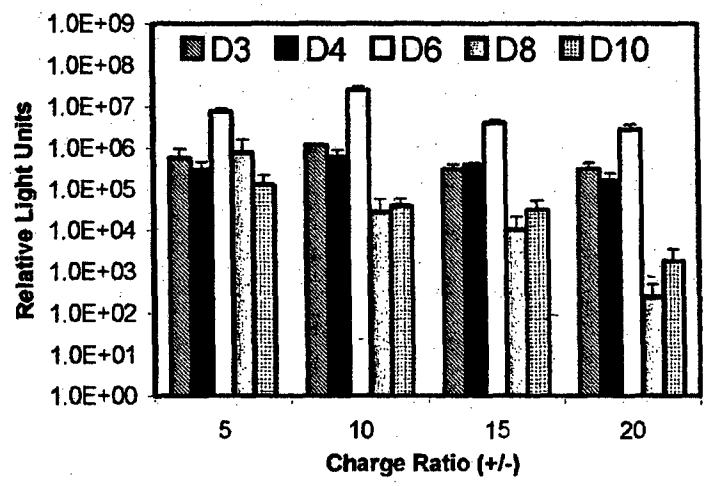


**Figure 4.** Comparison of transfection efficiencies of the DABCO polyplexes at charge ratios of 5, 10, 15, and 20 +/- with BHK-21 cells as determined by luciferase protein activity. Data are presented as a mean ± SD of three replicates.

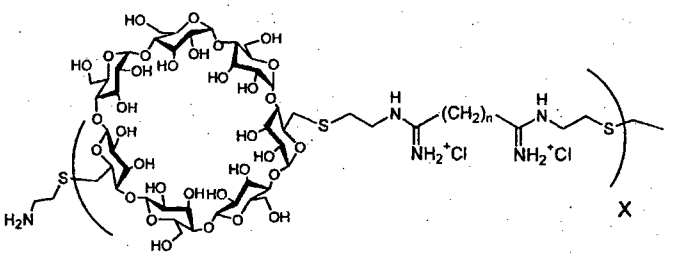

**Figure 5.** The structure of the βCDPs that were studied previously to elucidate structure-property relationships. The charge separation was varied by n = 4, 5, 6, 7, 8, and 10 methylene units between the amidine charge centers [6].

charge separation in polymeric vectors affects delivery efficiency and toxicity. These experiments also allowed for comparison of previous results regarding structure-property relationships that were examined using β-cyclodextrin polymers [6]. Here, several DABCO polymers were prepared and tested for their ability to bind and deliver pDNA in BHK-21 cells. Results indicated that all of the polymers studied bound pDNA and that the charge separation significantly affected both the transfection efficiency and the toxicity of these systems. The D3, D4, and D6 polymers were shown to have lower toxicity than the D8 and D10 polymers at charge ratios of 5, 10, and 15 +/-. In addition, the D6 polymer was consistently more efficient at gene delivery. This result coincides with previous findings in which the βCDP with 6 methylene units between the charges had the highest delivery efficiency. Further studies are currently being completed that are utilizing other models to more accurately elucidate the structure-property relationships for polycationic gene delivery vectors.

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